Effect of *E. faecalis* on the release of serine proteases elastase and cathepsin G, and collagenase-2 (MMP-8) by human polymorphonuclear leukocytes (PMNs)

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Abstract

Reynaud af Geijersstam A, Sorsa T, Stackelberg S, Tervahartiala T, Haapasalo M. Effect of *E. faecalis* on the release of serine proteases elastase and cathepsin G, and collagenase-2 (MMP-8) by human polymorphonuclear leukocytes (PMNs). *International Endodontic Journal*, **38**, 667–677, 2005.

Aim To measure the release of hydrolytic enzymes [elastase, cathepsin G and collagenase-2 (MMP-8)] from human polymorphonuclear leukocytes (PMNs) during interaction with strains of *Enterococcus faecalis* isolated from endodontic infections.

Methodology Six *E. faecalis* strains isolated from treatment resistant cases of apical periodontitis were included in the study. Overnight cultures of the microbes were used for the experiments. PMNs were isolated using the Ficoll Paque technique, and their vitality was assessed throughout the experiments by the Trypan Blue exclusion test. A known amount of microbes and PMNs were mixed in PBS at +37 °C in air, and the release of elastase, cathepsin G and MMP-8 was measured at 0, 20, 60 and 120 min after initiation of incubation. The activities of elastase and cathepsin G were analysed by spectrophotometer assays using specific synthetic peptide substrates, and MMP-8 by western immunoblotting quantitated by computer densitometry. PMNs incubated in buffer without any added microbes served as negative controls, cells incubated with 5 ng mL^{-1} phorbol myristic acetate (PMA) served as a positive control. The 95% confidence interval was used to compare the relative amount of elastase and cathepsin G released from the samples.

Results One *E. faecalis* strain induced a similar or higher elastase, cathepsin G and MMP-8 release than the positive control, whereas the other five strains induced only moderate or no release of the three enzymes examined as compared with the negative and positive controls. Western immunoblot revealed that released MMP-8 had molecular sizes of 60 and 75 kDa representing active and latent forms of MMP-8. In addition, >110 kDa high molecular size and a fragmented 20–30 kDa MMP-8 species could be observed.

Conclusions The majority of the *E. faecalis* strains induced little or no release of hydrolytic enzymes from the PMN cells. The finding may partly explain the clinical observation that root canal infections dominated by *E. faecalis* are usually symptom free.

Keywords: endodontics, enterococci, human polymorphonuclear leukocyte, MMP-8, serine proteases.

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Introduction

Presence of bacteria and their products in the apical region of an infected, necrotic dental root canal induces influx, activation and interaction of immune-inflammatory cells in the periapical area (Stashenko *et al.*

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1998). The successful mobilization of first line immune cells, including both specific and non-specific immune cells, prevents abundant extra-radicular bacterial invasion by effectively ingesting and killing the infective microorganisms, and thus opposes the spreading of the infection.

In chronic apical periodontitis, studies have shown polymorphonuclear leukocytes (PMN) to be the second most numerous cell present in chronic lesions after lymphocytes (Kontiainen *et al.* 1986, Stashenko 1998). PMNs are short lived-cells (5 days after leaving the bone marrow) and potent phagocytes. Whole bacterial cells and their virulence factors can induce enzyme release by selective degranulation of PMNs (Ding *et al.* 1997). PMNs are in general regarded as the most efficient first line of defence cells against invading microorganisms. Individuals with neutropenia are more prone to periodontal infections than individuals suffering some defects in the specific immunity, which causes decline in B or T-cell numbers (Stashenko *et al.* 1998). In chronic periapical lesions, which includes retreatment cases where *E. faecalis* is frequently present, a mixed infiltrate of specific and non-specific immune cells is present, a large proportion of these being PMNs (Kontiainen *et al.* 1986). PMNs are efficient phagocytes and release hydrolytic enzymes by degranulation facilitating their migration through inflamed tissue and preventing the spreading of infection. Elastase and cathepsin-G may act as inflammatory mediators both in chronic and acute inflammations (Selak 1994). In irreversible pulpal inflammation and apical periodontitis serine proteases such as elastase and cathepsin G are considered inflammatory markers (Cootauco *et al.* 1993). Increased levels of the collagenase matrix metalloproteinases-8 (MMP-8) in apical exudates have been reported in chronic apical periodontitis (CAP) (Wahlgren *et al.* 2002). These proteinases, especially in cascade, may cause significant host tissue destruction during the acute phase of an inflammatory process (Sorsa *et al.* 2004). Their involvement in periodontal tissue destruction is well documented (Ingman *et al.* 1996). MMP-8 is mainly produced by PMNs, suggesting an active role in apical tissue degradation for PMNs in CAP.

Enterococci are opportunistic bacteria capable of causing life-threatening infections in immunocompromized patients. They are one of the most frequently encountered nosocomial pathogens (Wisplinghoff *et al.* 2004) and have been isolated from urinary tract infections, endocarditis and bacteraemia (Richards *et al.* 2000). They are part of the human intestinal flora, and they are also used as starter strains in different foods (Franz *et al.* 2003). They possess a number of virulence factors, which may modify their ability to cause and maintain an infection.

Enterococcus faecalis is the most common endodontic pathogen in treatment resistant cases and root filled teeth with persisting infection (Molander *et al.* 1998, Peciuliene *et al.* 2001). Many of these cases are asymptomatic and remain undiscovered for years until a routine radiograph may reveal a periapical lesion (Peciuliene *et al.* 2000). Patients may have experienced recurrent mild pain from the affected tooth, whilst in other cases a flare-up may occur years after root canal treatment. Teeth with treatment-resistant apical periodontitis thus show a clinical status ranging from total absence of symptoms, to timely acute, purulent infections.

Clinical signs of an acute apical periodontitis (e.g. pain, abscess or a spreading infection) are a result of the interaction of the infective microorganisms with the host's defence system. The interaction between *E. faecalis* and PMNs has been studied primarily with respect to phagocytosis and killing of these microorganisms. The complement system plays a crucial role in the phagocytosis process of *E. faecalis* (Arduino *et al.* 1994) as does the presence of aggregation substance (AS), a sex pheromone produced by certain strains of *E. faecalis*, which binds to the same receptor as complement (Vanek *et al.* 1999).

Little is known about the stimulation and concomitant release of proteolytic enzymes from PMNs by *E. faecalis*. With this background, the aim of this study was to evaluate the release of several key proteolytic enzymes [elastase, cathepsin G and collagenase-2 (MMP-8)] from human PMNs triggered by different strains of *E. faecalis* isolated from endodontic infections.

Material and methods

Enterococcus faecalis isolates

Six clinical isolates of *E. faecalis* originating from root canals in teeth with treatment-resistant apical periodontitis were used. Identification of the isolates has been described earlier in detail (Sirén *et al.* 1997, Peciuliene *et al.* 2001). The isolates were cultured overnight at 37 °C on fresh horse blood agar plates [4.2% Brucella agar (Oxoid, Basingstoke, UK), 5 mg L^{-1} of Hemin (Fluca, Steinheim, Germany) 10 mL of menadione (Sigma, St Louis, MO, USA) and

5% defibrinated fresh horse blood]. One of the isolates was also cultured on Tryptic Soy Agar (Oxoid). The cells were then suspended in phosphate buffered saline pH = 7.3 (PBS) and adjusted to an optical density of 0.23 ± 0.01 at 450 nm corresponding to a concentration of 4×10^8 cells mL^{-1} as determined by culturing of serial dilutions of the suspension.

Isolation of PMNs

The PMNs were isolated using a modification of the Dextran/Ficoll method previously described (Segal *et al.* 1980). Briefly, 50 mL buffy coat (Blodbanken, Ullevål Hospital, Oslo, Norway) from a healthy donor was mixed with the same amount of an aqueous solution of 1% Dextran (Pharmacia Norge AS, Oslo, Norway) and 0.9% NaCl and left for sedimentation for 20–30 min. The erythrocyte-free layer was gently mixed with 20 mL Ficoll Paque (Pharmacia) and centrifuged at 350 *g* for 15 min. The supernatant was removed and pellet containing the PMNs was resuspended in 2 mL of PBS. The remaining erythrocytes were lysed by adding 10 mL ice cold sterile distilled water and gently vortex-mixed for 25 s. The reaction was stopped by immediately adding 12 mL ice cold hypertonic saline (1.8% NaCl) in order to restore isotonicity. The suspension was centrifuged at 350 *g* for 5 min and the supernatant removed leaving the pellet containing the PMNs.

The pellet was resuspended in 5 mL of Hank's balanced salt solution (HBSS) (Sigma) and the cells were counted in a Bürker chamber and adjusted to 1.05×10^7 cells mL^{-1}. The purity of the PMNs in this procedure is considered to be around 97% (Segal *et al.* 1980). The viability of the isolated PMN cells was tested by trypan blue staining on all 60- and 120-min samples by adding 1 : 1 volume of 0.4% trypan blue to an aliquot of these samples. Stained cells were considered dead.

E. faecalis and PMN interaction

An aliquot of 250 μL of the PMN suspension (1.05×10^7 cells mL^{-1}) was added to 250 μL of the bacterial suspension (4×10^8 cells mL^{-1}) and incubated at 37 °C for 0, 20, 60 and 120 min and then immediately placed on ice. The final concentration of PMNs was 5×10^6 cells mL^{-1}. The tubes were centrifuged at 350 *g* for 3 min in order to make the PMN cells sediment without breaking, and the supernatant was collected. As a positive control for PMN degranulation, 5 ng mL^{-1} phorbol myristic acetate (PMA, Sigma-Aldrich) was used, and as negative controls the

PMN suspensions were incubated with 250 μL of PBS or 250 μL of HBSS.

Elastase and cathepsin G

N-succinyl-Ala-Ala-Val *p*-nitroanilide (SAAVNA; Sigma) and *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-nitroanilide (SAAPNA; Sigma) were used as specific peptide substrates for the measurement of elastase and cathepsin G release from human leukocytes, respectively (Nakajima *et al.* 1979, Wenzel *et al.* 1980).

Fifty millilitre supernatants from the interaction reactions were defrozen and mixed with 10 μL of 10 mmol L^{-1} SAAVNA or 10 mmol L^{-1} SAAPNA and 40 μL TNC buffer (50 mmol L^{-1} Tris, 0.2 mol L^{-1} NaCl, 1 mmol L^{-1} CaCl2, pH 7.5) and placed in flat-bottom 96-well polystyrene ELISA plates at 37 °C. The absorbance was registered at 0, 1, 2, 3, 4 and 24 h at 405 nm on a Multiscan RC^{TM} ELISA reader (Labsystem, Helsinki, Finland) using Ascent Software v.2.6 (Thermo Electron Corporation, Waltham, MA, USA) and corrected for the background by subtracting the substrate/buffer control from the measured values. The enzyme activity was expressed in international units (IU) as the change of optical density per 0.001 min^{-1} (Ding *et al.* 1997). The 95% confidence interval of three parallel experiments was used to compare the relative amount of elastase and cathepsin G released from the samples, the hypothesis being that the average of the three measurements is the average of the normal distribution, the variance is unknown. The 95% CI was calculated according to the following formula: $x-k \leq \mu \leq x + k$, $k = (c\sigma)/\sqrt{n}$, x is the average, σ^2 is the variance of the measurements and c is the *t*-value for three samples with 2 d.f. ($c = 2.92$), and *n* is the sample size ($n = 3$) (Kreyszig 1999).

Western immunoblot for MMP-8

Western immunoblotting was used to detect molecular forms and degree of activation of MMP-8 utilizing specific anti-human-MMP-8 (Ding *et al.* 1996, 1997). MMP-8 analyses followed the Western immunoblot described for PMN supernatant by Tervahartiala *et al.* (2000) and Kiili *et al.* (2002). PMN supernatants were mixed with Laemmli's buffer and heated for 5 min at 100 °C, followed by protein separation with 8% SDS-polyacrylamide gels. The protocol for the ECL^{TM}-Western blotting analysis system was used according to the manufacturer's instructions (Amersham Pharmacia

Biotech, Little Chalfont, UK). We used specific polyclonal antibody for MMP-8 at a final concentration of 2 µg mL^{-1}, and quantitated the immunoblot by computer image scannings as previously described (Hanemaaijer *et al.* 1997, Apajalahti *et al.* 2003). Human PMN and rheumatoid synovial fibroblast culture media (Hanemaaijer *et al.* 1997, Kiili *et al.* 2002) served as positive controls for PMN- and fibroblast-type MMP-8. As Western blotting results are semi-quantitative, results from only one experiment representative of the three experiments are shown.

Results

Viability of the PMN cells

At 0 min less than 1% of PMN cells were dead, and at 60 min 14 ± 9.6% of all PMN cells were dead (16% of PMNs incubated with bacteria, 15% of PMNs in the positive control and 10% of PMNs in the negative controls). At 120 min 15 ± 9.3% of the PMNs were dead (17% of PMNs with bacteria, 12% of PMNs incubated with PMA and 7% of PMNs in the negative controls).

Release of elastase and cathepsin G

The activities measured as variation of optical densities per 0.001 min^{-1}, of the three parallel measurements and their 95% confidence intervals are shown in Table 1 and Fig. 1 for elastase, and Table 2 and Fig. 2 for cathepsin G. One of the six *E. faecalis* strains caused release of elastase from PMNs in elevated amounts. The activity measurements at 60 min of all samples other than Gel1-31 are outside the 95% confidence intervals of sample Gel1-31 for the respective incubation-times with one exception (vp3-69, 60 min, one of three). The 95% confidence interval of Gel1-31 is above or within the 95% confidence interval of the positive control for the elastase measurements. A lower cathepsin G activity than the positive control with PMA was registered from all the samples. No significant difference in activation of PMNs was found for sample Gel1-32, when cultured on both blood agar versus TSA.

Release of MMP-8 by PMN cells after interaction with E. faecalis

At 120 min, similar amounts of MMP-8 was measured by western blot analysis of the supernatants from PMNs

Table 1 Elastase activities in *E. faecalis* triggered PMN supernatants measured as variation of optical densities per 0.001 per minute and corresponding 95% confidence intervals (CI)

Sample	0 min activities	20 min activities	95% CI From	95% CI To	60 min activities	95% CI From	95% CI To	120 min activities	95% CI From	95% CI To
vp2 77	0.151; 0.159; 0.134	0.313; 0.480; 0.488	0.127	0.170	0.261	0.594	0.222; 0.330; 0.326	0.189	0.396	0.213; 0.205; 0.372	0.105	0.422
gel1 31	0.267; 0.209; 0.113	1.651; 0.955; 1.780	0.065	0.328	0.714	2.210	1.072; 1.888; 1.634	0.827	2.236	1.755; 2.001; 1.568	1.408	2.141
gel1 32	0.188; 0.172; 0.201	0.243; 0.380; 0.376	0.162	0.212	0.201	0.465	0.393; 0.359; 0.272	0.236	0.446	0.298; 0.463; 0.355	0.225	0.516
vp3 69	0.147; 0.209; 0.168	0.322; 0.597; 0.547	0.121	0.228	0.241	0.735	0.272; 0.438; 1.280	−0.248	1.575	0.284; 0.368; 0.176	0.114	0.438
vp3 72	0.109; 0.118; 0.143	0.168; 0.543; 0.193	0.094	0.152	−0.053	0.654	0.172; 0.184; 0.255	0.128	0.279	0.205; 0.197; 0.172	0.162	0.220
vp3 79	0.130; 0.105; 0.143	0.226; 0.276; 0.276	0.094	0.158	0.211	0.308	0.351; 0.497; 0.580	0.280	0.671	0.347; 0.305; 0.401	0.270	0.432
gel1 32*	0.305; 0.147; 0.226	0.438; 0.372; 0.313	0.092	0.359	0.269	0.480	0.376; 0.447; 0.322	0.276	0.487	0.384; 0.268; 0.663	0.095	0.781
Positive control PMA	0.209; 0.197; 0.330	0.401; 0.834; 0.538	0.121	0.369	0.218	0.964	0.888; 0.797; 1.101	0.666	1.192	3.263; 3.284; 2.751	2.590	3.609
Negative control HBSS	0.130; 0.159; 0.018	0.343; 0.218; 0.355	−0.024	0.228	0.177	0.433	0.218; 0.168; 0.338	0.093	0.389	0.147; 0.155; 0.272	0.073	0.309
Negative control PBS	0.163; 0.105; 0.009	0.201; 0.209; 0.222	−0.039	0.224	0.193	0.228	0.197; 0.276; 0.551	0.028	0.654	0.251; 0.234; 0.180	0.159	0.284

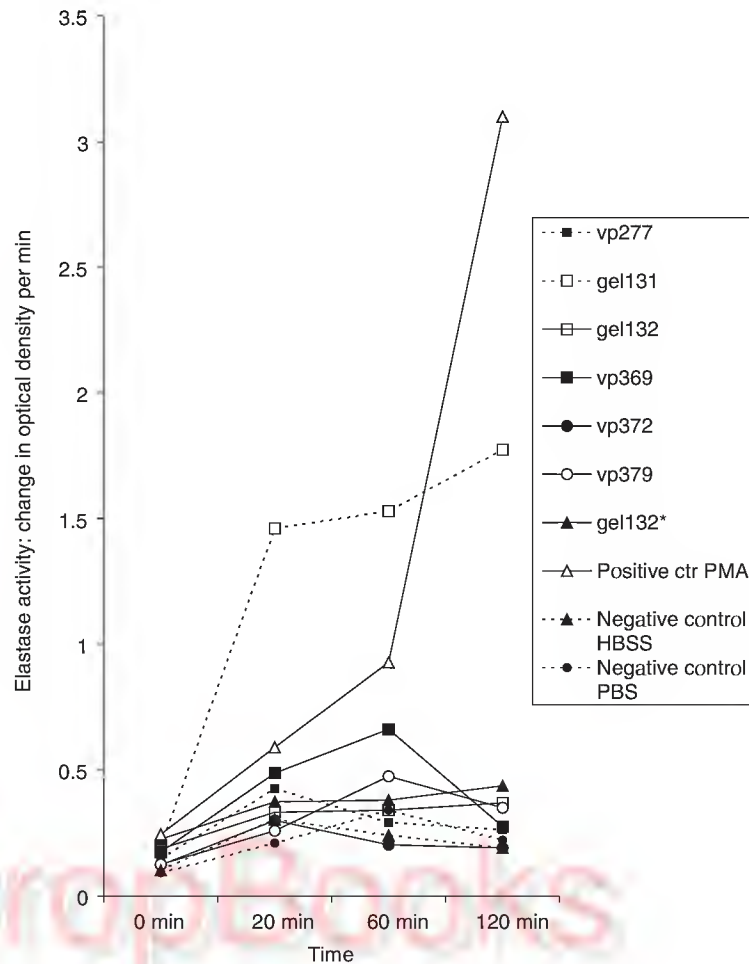*It was grown on tryptic soy agar, all other samples were grown on blood agar.

Figure 1 Average time dependent release expressed in international units of elastase by PMNs triggered with various strains of *E. faecalis* and phorbol myristic acetate (PMA) and negative controls with phosphate buffered saline (PBS) and Hank's balanced salt solution (HBSS). The name of each sample is given in the key.

incubated together with *E. faecalis* strain gel1-31 and the positive control with PMA. All the other 120 min samples were below 50% of the intensity of the positive control (Table 3 and Fig. 3). Western immunoblot revealed that released MMP-8 had molecular sizes of 60 and 75 kDa representing active and latent forms of PMN-derived MMP-8. In addition high (>110 kDa) molecular size and fragmented 20–30 kDa MMP-8 species could be detected (Fig. 4).

Discussion

Although PMNs mainly protect us from infections, a consequence of PMN activation is that host tissue is also destroyed co-operatively by serine proteases, such as elastase, cathepsin-G and MMPs (Weiss 1989, Sorsa *et al.* 2004). Interestingly, an anti-inflammatory role during acute lung injury in mice has recently been

suggested for MMP-8 (Owen *et al.* 2004), and the overall role of MMP-8 in acute and chronic apical periodontitis is not fully understood yet (Sorsa *et al.* 2004).

A root canal infection stimulates immune cells to migrate to the periapical area. The ability of bacteria to stimulate PMN migration, activation, phagocytosis and killing is one measure of their virulence. The role of complement in phagocytosis of *E. faecalis* has been established, as well as the presence of anti-enterococcal antibodies in phagocytic killing of *E. faecalis* (Arduino *et al.* 1994). Aggregation substance has also been shown to promote non-opsonic binding to PMNs using the complement three receptor as a binding site on the PMNs (Rakita *et al.* 1999). The same authors later found that AS-binding to PMNs and consequent phagocytosis protects *E. faecalis* from being killed, despite activation of PMNs measured as expression

Table 2 Cathepsin G activities measured as variation of optical densities per 0.001 per minute and corresponding 95% confidence intervals (CI)

Sample	0 min activities	95% CI From	To	20 min activities	95% CI From	To	60 min activities	95% CI From	To	120 min activities	95% CI From	To
vp2 77	0.063; 0.096; 0.079	0.051	0.107	0.133; 0.083; 0.108	0.066	0.150	0.163; 0.108; 0.121	0.083	0.178	0.088; 0.129; 0.158	0.065	0.185
gel1 31	0.133; 0.121; 0.079	0.063	0.159	0.129; 0.100; 0.150	0.084	0.169	0.200; 0.221; 0.217	0.194	0.231	0.192; 0.200; 0.229	0.174	0.240
gel1 32	0.125; 0.104; 0.075	0.059	0.144	0.113; 0.092; 0.113	0.085	0.126	0.150; 0.117; 0.108	0.088	0.162	0.079; 0.129; 0.208	0.029	0.249
vp3 69	0.142; 0.113; 0.008	−0.031	0.206	0.158; 0.058; 0.075	0.007	0.188	0.313; 0.146; 0.167	0.055	0.361	0.117; 0.163; 0.154	0.103	0.186
vp3 72	0.054; 0.063; 0.029	0.019	0.078	0.096; 0.067; 0.092	0.058	0.111	0.108; 0.113; 0.117	0.105	0.120	0.104; 0.117; 0.104	0.096	0.121
vp3 79	0.067; 0.088; 0.050	0.036	0.100	0.092; 0.117; 0.142	0.075	0.159	0.196; 0.171; 0.221	0.154	0.238	0.133; 0.233; 0.379	0.040	0.457
gel1 32*	0.075; 0.104; 0.063	0.045	0.117	0.146; 0.338; 0.188	0.091	0.148	0.313; 0.117; 0.229	0.054	0.385	0.129; 0.254; 0.200	0.089	0.300
Positive CTR PMA	0.075; 0.104; 0.063	0.059	0.094	0.146; 0.338; 0.188	0.054	0.394	0.429; 0.263; 0.296	0.180	0.478	0.433; 0.525; 0.529	0.405	0.587
Negative control HBSS	0.088; 0.067; 0.075	−0.039	0.131	0.075; 0.121; 0.117	0.061	0.147	0.038; 0.075; 0.013	−0.011	0.095	0.108; 0.075; 0.092	0.064	0.120
Negative control PBS	0.075; 0.067; 0.000	−0.022	0.117	0.054; 0.092; 0.092	0.043	0.116	0.133; 0.188; 0.167	0.116	0.209	0.092; 0.071; 0.092	0.064	0.105

*It was grown on tryptic soy agar, all other samples were grown on blood agar.

of surface proteins on the PMNs and production of extracellular superoxide and phagosomal oxidant (Rakita *et al.* 1999).

In the present study, we examined activation of PMNs by measuring the release of the serine proteases elastase, cathepsin G and collagenase-2 (MMP-8), when incubated with a selection of endodontic strains of *E. faecalis*. Elastase and cathepsin G are contained in the azurophil granules and MMP-8 in specific granules of PMNs (Borregaard & Cowland 1997). Serum free PMNs were used and thus incubated non-opsonized bacteria with the PMNs, in order to eliminate possible interactions of serum molecules with PMNs and microbial cells. Serum contains several constituents, i.e. α_2-macroglobulin, serpins and tissue inhibitors of matrix metalloproteinases (TIMPs) (Cawston *et al.* 1986), which are likely to interfere with the measurement of PMN enzymes after they have been released.

The cathepsin G activity of the positive control (PMA stimulation) was significantly higher than the negative controls for samples taken at 60 and 120 min. The activities of the samples taken earlier than 60 min all overlapped with the negative controls, thus no difference in secretory stimulation could be demonstrated. With one *E. faecalis* strain (gel1-31), the 60-min activity values for both cathepsin-G and elastase were within or above the 95% CI of the positive control, indicating a significantly higher activation potential for this strain compared with the other isolates. The results of cathepsin-G release (Table 2) showed that none of the samples had all three parallel results within the 95% CI of the positive control for 120 min, but for strain gel1-31, vp3-79 and gel1-32 on TSA plates, all values from the three parallel experiments were above the 95% CI of the negative controls, suggesting a moderate induction of cathepsin G secretion after 120 min of incubation. Gel1-31 induced a level of MMP-8 secretion corresponding to the level of the positive control (PMA) at 120 min, again showing a potential for activation of this sample compared with the other isolates tested. The released MMP-8 species were shown to represent 60 kDa active form and 7s kDa latent proform of PMN-derived MMP-8. In addition, ≥110 kDa high molecular size and 20–30 kDa fragmented MMP-8 species (sample vp2-69, 60 min, Fig. 4) may also, at least in part, represent shedded forms of defensive or anti-inflammatory membrane-bound MMP-8 (Owen *et al.* 2004). Western blotting being a semi-quantitative method, these results only give an indication of activation of PMNs with respect to MMP-8. Ding *et al.* (1997) in a similar
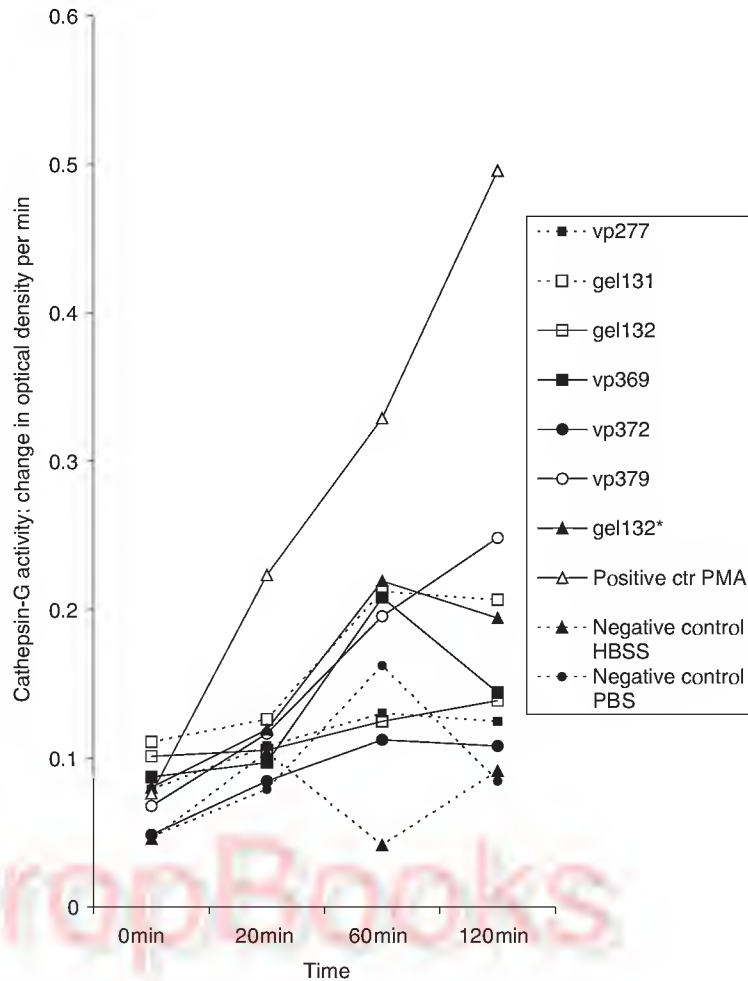
Figure 2 Average time dependent release expressed in international units of cathepsin G by PMNs triggered by various strains of *E. faecalis* and phorbol myristic acetate (PMA) and negative controls with phosphate buffered saline (PBS) and Hank's balanced salt solution (HBSS). The name of each sample is given in the key.

Table 3 Densitometrical analysis of the total immunoreactivity of MMP-8 released by PMNs expressed in an arbitrary unit

Samples	0 min	20 min	60 min	120 min
vp2 77	0	0	0.291	2.282
gel1 31	0	0.514	0.948	6.064
gel1 32	0	0.009	0	2.602
vp3 69	0	0	0.061	2.602
vp3 72	0	0	0.155	2.565
vp3 79	0	0	0.743	0.734
gel1 32*	0.893	2.205	1.6581	3.042
Positive CTR PMA	0.692	1.627	5.762	6.099
Negative control HBSS	0	1.945	0.925	2.006
Negative control PBS	0.688	0.513	0.543	0.151

*It was grown on tryptic soy agar, all other samples were grown on blood agar.

experimental setting with *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Treponema denticola* recorded no cathepsin G activity, whereas elastase activity

was strongly elevated by *F. nucleatum*. It has been demonstrated that exocytosis of azurophilic granules in PMNs happens simultaneously regardless of their content (Faurschou *et al.* 2002). Cathepsin G and elastase activities at the 120 min samples of Gel1-31 were significantly lower than the corresponding activities released by PMNs stimulated with PMA. This could be because of the elimination of bacteria by phagocytosis by PMNs leading to reduced activation of PMNs and/or to extracellular degradation of cathepsin G and elastase by the bacteria. Degradation or inhibition by traces of serum serine protease inhibitors such as α1-antitryspin, α2-macroglobulin, α1-antichymotrypsin and the secretory leukoproteinase inhibitors (SLPI) was controlled for by the positive control (PMA). Faurschou *et al.* (2002) observed that after 15 min of stimulation of PMN cells by PMA, only a moderate degranulation of azurophilic granules was obtained as
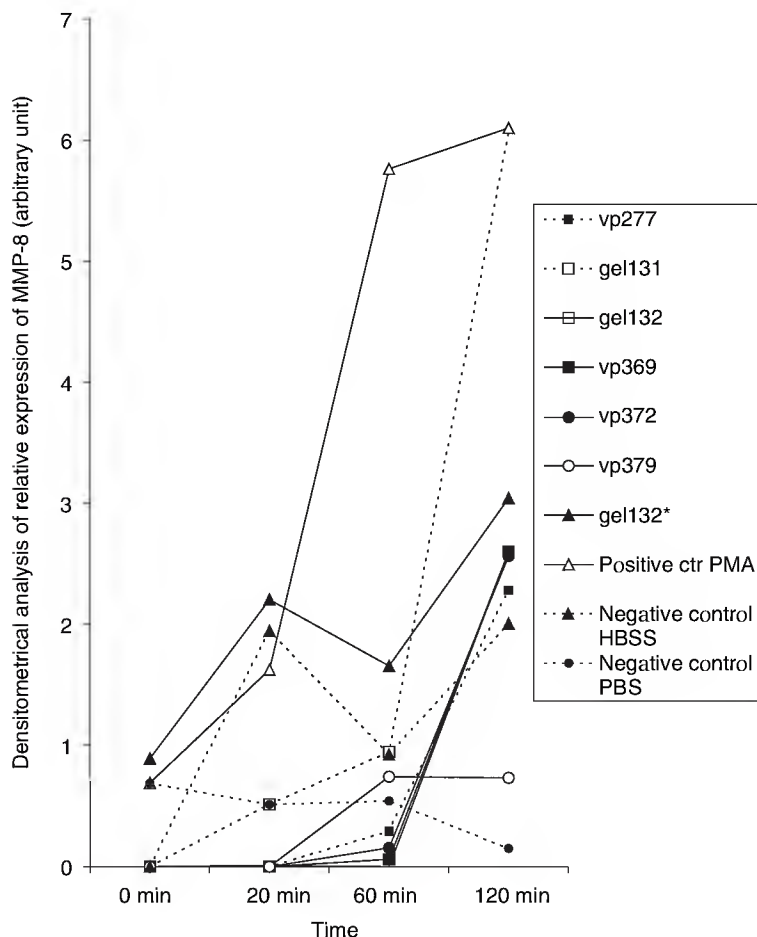
Figure 3 Densitometrical analysis of the total immunoreactivity expressed in an arbitrary unit of MMP-8 by PMNs triggered by various strains of *E. faecalis* and phorbol myristic acetate (PMA) and negative controls with phosphate buffered saline (PBS) and Hank's balanced salt solution (HBSS). The name of each sample is given in the key.

compared with ionomycin or cytochalasin. Elastase activity induced by gel1-31 was significantly higher than the positive control at 20 and 60 min. This may be because of the fact that total degranulation of serine proteases by PMNs is not maximal at 60 min, when stimulated by PMA. Nevertheless, elastase and cathepsin G activities induced by PMA increased to significantly higher levels relative to all tested *E. faecalis* strains, indicating that PMA stimulation continued throughout the experiment and exceeded that of each tested *E. faecalis* strain.

In the context of endodontic infections, *E. faecalis* is typically isolated from asymptomatic treatment resistant-infections, and it often occurs as a mono-infection (Molander *et al.* 1998, Peciuliene *et al.* 2001). It has been reported (Peciuliene *et al.* 2001) that the only two cases of a flare-up after retreatment of 40 root filled teeth occurred when *E. faecalis* had previously been isolated together with either *F. nucleatum* and *Actinomyces viscosus*, or *Proteus mirabilis*, which were the major

isolates. Both *F. nucleatum* and *A. viscosus* are well known abscess-forming anaerobes often isolated from dental abscesses (Dahlén 2002). *Proteus mirabilis* appears not to have been reported in dental abscesses, but was the major isolate and is therefore likely to cause the flare-up. Möller *et al.* (2004) showed in an experimental model on monkeys that *E. faecalis*, when inoculated into the devitalized pulp together with *Streptococcus milleri*, *Peptostreptococcus anaerobius*, *Prevotella oralis*, *Bacteroides oralis* and *F. nucleatum*, could be reisolated alone or in different combination with the above bacteria in all radiologically diagnosed apical periodontitis cases before endodontic treatment, and was a strong survivor after a two-session endodontic therapy, with an isolation rate of 75% after two completed sessions of endodontic therapy. Others have shown *in vitro* and *in vivo* that these organisms are resistant to most root canal medicaments and are well adapted to the nutrient poor milieu of the root canal (Peciuliene *et al.* 2001, Portenier *et al.* 2001). It seems
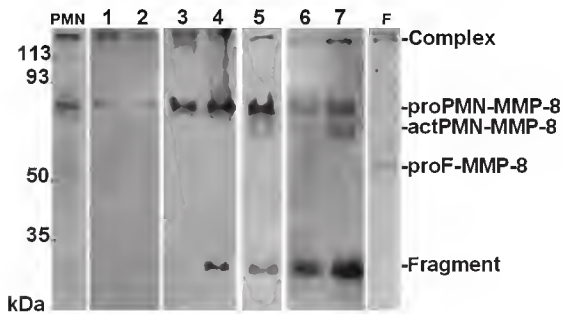
Figure 4 Western immunoblot analysis of MMP-8 released by PMNs exposed to *E. faecalis*. Lanes 1 and 2: PMN incubated in buffer for 20 and 60 min, respectively; lanes 3 and 4: *E. faecalis* gel1-31 for 20 and 60 min, respectively; lane 5: PMA for 60 min, lanes 6 and 7: *E. faecalis* vp3–69 for 20 and 60 min, respectively. PMN indicates human neutrophil culture media and F indicates human rheumatoid synovial fibroblast culture media used as positive controls for PMN-type and fibroblast-type MMP-8, respectively. Complex indicates high >110 kDa molecular size MMP-8 species; proPMN-MMP-8 indicates neutrophil-type pro-MMP-8; actPMN-MMP-8 indicates neutrophil-type active MMP-8; proF-MMP-8 indicates fibroblast-type pro-MMP-8, and fragment indicates low molecular weight size truncated MMP-8 species. Mobilities of the molecular weight markers are indicated on the left.

that *E. faecalis* is causing or maintaining a chronic infection in the root canal, but it is seldom associated with acute endodontic infections (Haapasalo *et al.* 2003). The fact that only one of the tested *E. faecalis* strains stimulated release of MMP-8, elastase and cathepsin G by the PMNs in the range of the positive control supports the clinical observations that few of these infections give rise to acute clinical inflammatory symptoms (i.e. large bone destruction, pain, tenderness to percussion or abscess formation). Fabricius *et al.* (1982) reported that *E. faecalis* in mono-infections seldom caused strong apical responses, but in combination with other species (*B. oralis*, *S. milleri*, *F. nucleatum* and others), resulted in larger apical bone destruction. It is also interesting to note that one strain (gel1-31) induced a higher inflammatory proteolytic response relative to our positive control (PMA).

In the early stages of an endodontic infection the microbial flora is dominated by gram negative anaerobic bacteria, but the changing ecological pressure during treatment in chronic cases will favour the establishment and persistence of facultative gram positives such as *E. faecalis* (Sundqvist 1992). Thus, the periapical bone resorption during the acute phase of the infection may be followed by a quiet phase with an infective flora dominated by *E. faecalis*. These results

also indicate that a microbiological diagnosis to species level may not be a sufficient indicator of the virulence of the isolated strains, as for *E. faecalis* certain strains are clearly more virulent than others. Different virulence factors have been identified in *E. faecalis* which may contribute to its ability to colonize and persist in the root canal (Hubble *et al.* 2003). These factors may also contribute to resistance to phagocytosis.

Conclusions

The results of the present study indicate a variable potential for different endodontic *E. faecalis* strains to induce proteinase release from PMNs. Only one of the six strains of *E. faecalis* used in the study induced considerable PMN degranulation. This possibly reflects the clinical observation that endodontic retreatment cases seldom show acute inflammatory reactions. These findings emphasize the need for well characterized *E. faecalis* strains when studying interactions between host defence protagonists and these bacteria. The clinical significance of the results could be further evaluated by analysing the enzyme activity of inflammatory exudates in *E. faecalis* infected root canals with different symptoms.

Acknowledgements

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